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INDO AMERICAN JOURNAL OF
PHARMACEUTICAL SCIENCES

Available online at: http://www.iajps.com *Review Article*

BIOANALYTICAL METHOD DEVELOPMENT AND METHOD VALIDATION IN HUMAN PLASMA BY USING LC MS/MS

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Article Received: December 2018 **Accepted:** February 2019 **Published:** March 2019

Abstract:

Bioanalytical method development plays importance role in the pre-clinical and clinical studies. Pharmacokinetics of any drug and its metabolite can be recognized by bioanalytical studies. The quantitative analysis of drugs and their metabolite sin the biological media is done by bioanalytical studies. Physical-chemical and biological techniques are used for these studies. Every bioanalytical method should be selective, sensitive and reliable for the quantitative estimation in drug discovery process. Bioanalytical method development consists of sample preparation, chromatographic separation and detection by using proper analytical method. Each developed method should be validated as per the regulatory authorities, so as to give reliable and reproducible method for the intended use. Many analytical techniques can be used for bioanalysis; LCMS/MS is one of them. In Liquid chromatography-mass spectrometry [LC-MS/MS] the separation of analyte is done by LC and detection is carried out by MS. LC-MS/MS obviously used in estimation and understanding of bioavailability, bioequivalence and pharmacokinetic data. This review additionally centered on different validation parameters such as: accuracy, precision, sensitivity, selectivity, standard curve, limits of quantification, range, recovery stability, etc.

Keyword: *Solid phase extraction, Liquid-Liquid Extraction, cartridge, LC MS/MS, Bioanalysis, Validation.*

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Please cite this article in press Rahul Patil et al., ***Bioanalytical Method Development And Method Validation In Human Plasma By Using Lc Ms/Ms.,*** *Indo Am. J. P. Sci, 2019; 06[03].*

INTRODUCTION:

Bioanalytical Methods are broadly engaged for the quantitative analysis of the drugs.[1] High penetrating and ultra-fast high performance liquid chromatography and tandem mass spectrometry [LC–MS/MS] assay is developed and validated for the quantification in human plasma.[2] Bioanalysis is the method to study the concentration of analyte, their metabolites and endogenous elements in biological matrix's such as blood, plasma, serum, urine, and saliva. The situation also plays an main role in the evaluation of bioavailability, bioequivalence, pharmacokinetics studies.[1]

Pre-clinical and clinical department support the bioanalytical process. Pre-clinical regulatory including Good Laboratories Practices [GLP]. Tuned to compound requires extensive method development but could leverage preclinical methods. Validation strategy is provided in clinical department in bioanalysis. Clinical and Non-clinical bioanalytical method development [MD] activities do not mandatory to be completed in compliance with GLPs however sufficiently documented to support a reproducible method document intended for validation.[3] Analytical techniques utilized for the quantitative assurance of analyte and their metabolites in biological samples are the key determinants in generating reproducible and reliable information data which thus are utilized in the assessment and understanding of bioavailability, bioequivalence, and pharmacokinetic discoveries [4].

LC-MS/MS is an amazing scientific procedure for quantitative bioanalysis because of its inherent high sensitivity and selectivity. It is susceptible, be that as it may, to matrix effects. The effect of lattice impacts on the accuracy, precision and robustness of bioanalytical methods is of increasing concern in the pharmaceutical company. The majority of the above components can cause critical errors in the accuracy and precision of bioanalytical procedures. Present FDA guidance documents currently need that this impact be evaluated as a part of quantitative LC/MS/MS method development, validation and routine use. The sample preparation and chromatographic methods for their adequacy in reducing or removing matrix effects, important to the greatest robust, sensitive assay possible.[5]. Liquid chromatography [LC] coupled by an atmospheric pressure ionization source to tandem mass spectrometric [MS/MS] detection is as of now considered as the method of excellent for quantitative analysis of compounds in biological matrixes. The benefits of utilizing MS/MS in the selected reaction monitoring mode are mostly the improved specificity, sensitivity.[6]

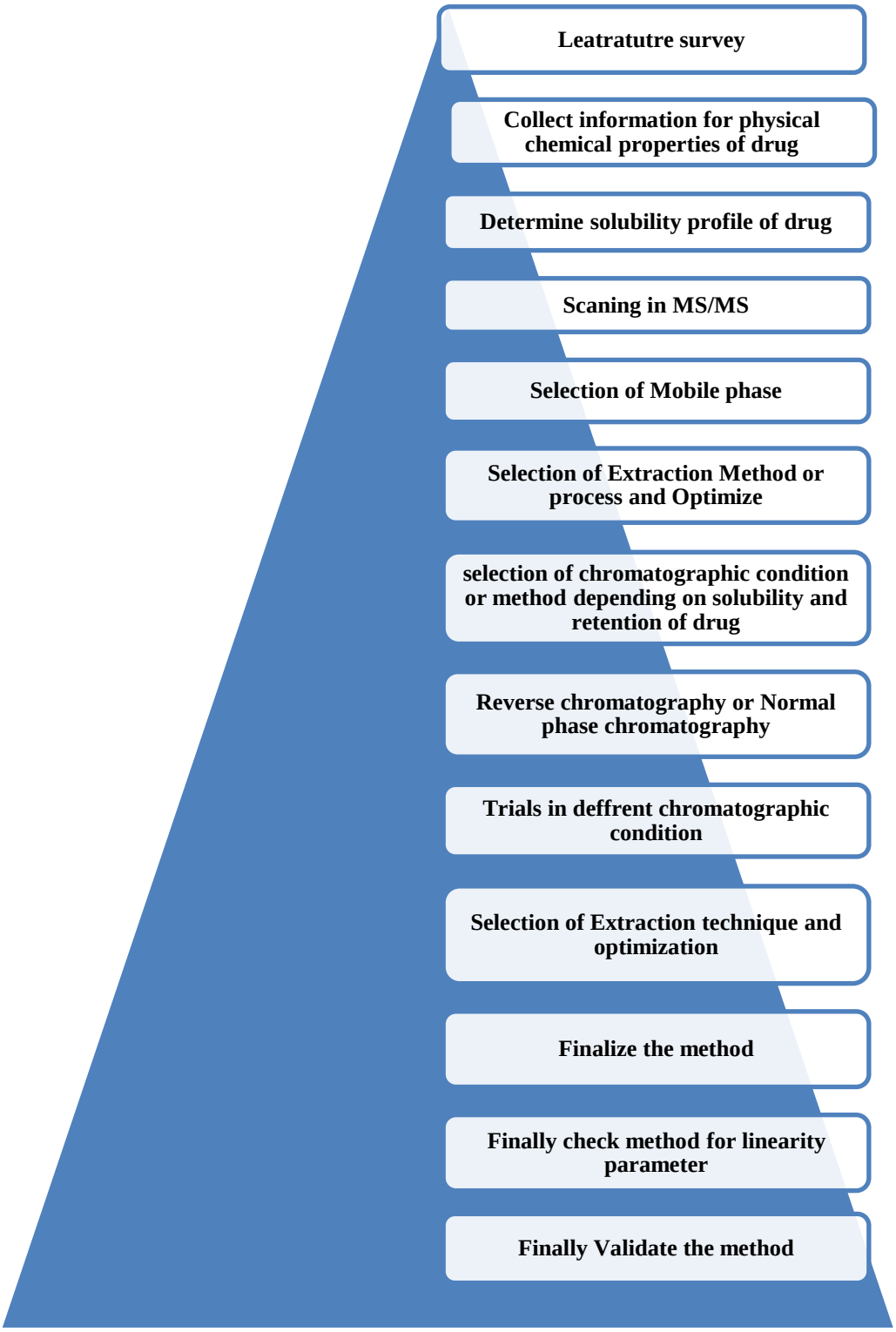
Bioavailability is characterized as the rate and degree to which the active component or active moiety is ingested from a drug and becomes accessible at the site of action. For drug products that are not intentional to be absorbed into the bloodstream, bioavailability might be surveyed by estimations planned to duplicate the rate and degree to which the active components or active moiety winds up obtainable on the site of action. This definition centers around the procedures by which the active components or moieties are discharged from a dosage form and travel to the site of action.[7]

Bioequivalence is characterized as "The nonappearance of a significant alteration in the rate and degree to which the active components or active moiety in pharmaceutical counterparts becomes obtainable at the site of drug action when managed at the similar molar dose under comparable situations in an properly designed study."[7]

METHOD DEVELOPMENT:

Bioanalytical method development activities are not required to be performed in consistence with GLPs however ought to be satisfactorily documented to help a reproducible method document for validation. Method development is intended to define the method and provide sound scientific evidence for method design and suitability for its intended purpose.[8]The method development under search literature and identification physical, chemical properties of drug. Mass spectrometry was a fairly routine tool for molecular weight determination and a specialty tool for complex structure identification problems. The survey of literature checking pharmacokinetic profile, identification of the structure , molecular weight, molecular formula, drug dose, protein binding and solubility. The method development is identification of analytical technique.[9] Analytical technology leads to improvements in sensitivity & selectivity. Selection of tools based on nature of analytes, analytical sensitivity, selectivity etc, sample matrix & sample stability. In addition, stability of analytes needs to be considered from time of sample collection through final analytical measurement.[3] The reliability of the method was assessed based on linearity, sensitivity, selectivity, precision and accuracy, recovery and carry over effect. Based on the results acquired for the above parameters.[2]

Flow chart of method development



Drug Extraction Techniques:
Method of extraction is the important role play in bioanalytical method development and validation.

Solid Phase Extraction:
Sample preparation is an essential module of analytical techniques. It is used to concentrate an drugs to increase its limits of detection, as well as to separate an analyte from undesirable matrix components that can cause interferences upon analysis.[10] Solid phase extraction [SPE] is an extraction technique based on the selective partitioning of one or more compounds between two phases, one of which is a solid sorbent. Solid phase extraction [SPE] is an extraction method that uses a solid and liquid phase to separate one, or more type, of drug from a solution and is used to remove certain compounds from a mixture of impurities based on their physical and chemical properties[11].The SPE better than other type of sample preparation technique such as, LLE [Liquid- Liquid Extraction] and protein precipitation. SPE [Solid Phase Extraction] is an effective technique, often achieving higher recovery of analyte than other methods of sample preparation for the reason that of selectivity. The chemistry of attraction between an analyte and the solid sorbent can be demoralized by pH and solvent considerations to allow interaction yet eliminate interventions.[10] . In SPE small plastic expandable column or cartridge pressed with 0.1 to 0.5 g of sorbent which is generally RP material [C18 or C8] is utilized. The analyte may either specially adsorbed to the strong, or they may stay in the liquid phase.[1]

Step of Solid Phase Extraction

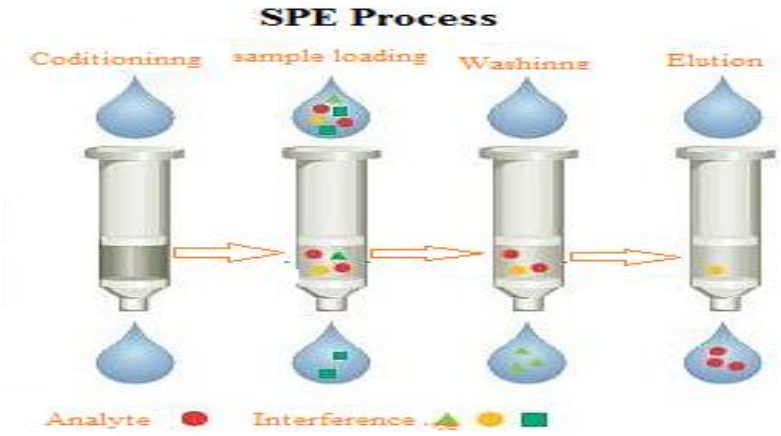
1) **Conditioning**
2) **Loading**
3) **Washing**
4) **Elution**

1) **Conditioning**
Conditioning is normally activation of the column. The organic solvents perform as a wetting agents. The organic solvent are used in drug related functional group. For proper adsorption 5% methanol and water is added the activation of column.

2) **Loading**
The aliquot sample is loaded in cartridge.

3) **Washing**
After the sample loading, wash the column. Matrix is remove and drug remain.

4) **Elution**
Elution for the suitable organic solvent or buffer used. Which elute the drug from the SPE analysis.[12]



TYPE CARTRIDGE:

1) Reversed Phase Extraction

Reversed phase partitions include a polar [water] or decently polar sample matrix [versatile phase] and a nonpolar stationary phase. The analyte of intrigue is ordinarily mid-to non-polar. its knowledge to most liquid chromatographers, the number and variability of profitable phases available, and the large number of published applications. Reversed-phase chromatography can be utilized to isolated nonpolar, polar, and ionic compounds some of the time in a similar detachment. The technique also can complete a upper range of separations than all other modes combined since it allows users to operate the mobile phase by changing organic solvent type, solvent composition, and pH.

Polymeric materials such as poly styrene–divinylbenzene [PS–DVB] discovery certain usage as reversed-phase media [for Eg, with high-pH mobile phases] but commonly arrange for lower column efficacies than those of silica gel–based packing. Water, normally buffered, blended with a water-miscible organic dissolvable [solvent, modifier], most generally acetonitrile or methanol, is the favored mobile phase. Reversed phase chromatography usually is performed using octadecyldimethylsilane [C18] or octyldimethylsilane [C8] stationary phases bonded to high-purity, spherical silica gel.[13]

2) Normal Phase extraction

Normal phase SPE strategies regularly incorporate a polar analyte, a mid to nonpolar matrix [e.g. acetone, hexane, chlorination solvents], and a polar stationary stage. Retention of an analyte under ordinary phase conditions is predominantly because of interactions among polar functional groups of the analyte and polar groups on the sorbent surface.[14]

3) Ion Exchange extraction

The SPE can be used in compound charge in specific solution but sometime organic solution. Ion suppression of drug response is a main source of in accuracy for bioanalytical analysis by using LC–MS/MS. Endogenous phospholipids cause ion suppression in both positive ESI and negative ESI modes and fundamental to be settled [resolved] chromatographically.[14]

1) Anion Exchange: The ion are negatively charged and compound are isolated on SAX NH_2 bonded silica. The SAX material contains aliphatic amine group is bonded in silica base surface.[15]

2) Cation Exchange: The ions are positively charged and compound are isolated on SCX or WCX bonded silica. The SCX material contains aliphatic sulfonic acid group bonded in silica base surface.[14]

Liquid-Liquid Extraction

The distribution of a solute depends on its preference for one or the other liquid, which is closely related to its solubility in each one of them. The common subject of solubilities is very high relevant to solvent extraction.

Many solvent are used in liquid-liquid extraction. They can be divided into five different classes as follows

Class- 1 liquid capable of forming three dimensional of strong hydrogen bonds, e.g; water, poly-amino alcohol, hydroxyl acid etc.

Class- 2 Other liquids that have both active hydrogen atom and donor atoms, but do not form three dimensional network [rather forming chainlike oligomers]. E.g. primary alcohols, carboxylic acid, primary and secondary amine, nitro- compounds, liquefied ammonia etc. They are generally called protic or protogenic substances.

Class- 3 Liquid collected of molecules containing donor atoms, but no active hydrogen atoms, e.g. ether, ketones, aldehydes, esters, tertiary amines, nitro compounds without α-hydrogen.

Class- 4 Liquid composed of molecules containing active hydrogen atoms but no donor atoms. e.g. chloroform and some aliphatic halides.

Class-5 Liquid with no hydrogen bond forming capability and no donor atoms, e.g. hydrocarbons, carbon disulfide, carbon tetrachloride. [13]

Protein Precipitation

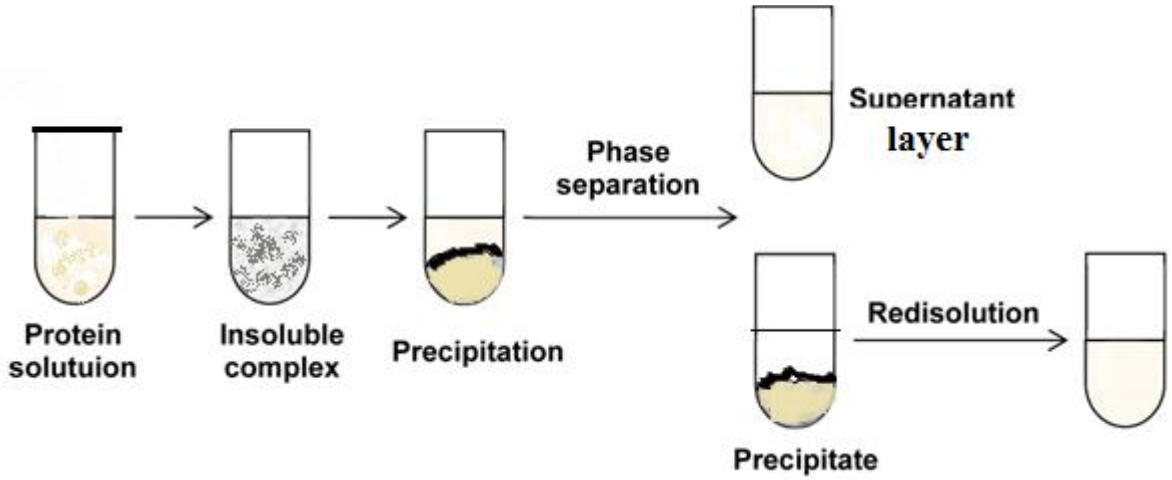
Protein precipitation is normally used for rapid sample clean-up and the disturbing protein drug binding.

Four categories of protein precipitation techniques there are follows

1) Organic solvent
2) Acid
3) Salt
4) Metal ion

Solubility results from polar interactions with aqueous solvent, ionic interactions through salts & repulsive electrostatic forces between like charged molecules. Precipitants apply exact effects on

proteins to enable their precipitation from liquid. Natural solvent [organic solvent] precipitant reduction the dielectric gone of the plasma protein solution, which growths the attraction between charged molecules and allows electrostatic protein interactions. The organic solvent similarly moves the orderly water molecules about the hydrophobic areas on the protein surface.[16]



Stationary Phase selection
The stationary phase is depending on some following parameter.

Column selection
Column is heart of separation process and they serious impact on method development and validation. Selection of column based upon knowledge of sample and goals of separation. The various factors including column selection follow as

Diameter of Column
Generally narrow diameter column is more sensitive so wider diameter column select greater sample loading.

Particle size
Smaller particle- 3-4µm
Bigger particle- 5-10µm

Selection of mobile phase
Mobile phase is play important role in improving the peak area [Resolution] and improving peak sensitivity. In choose the rights match among stationary phase and mobile phase compositions, decrease total time of analysis and increase peak height.

Buffer
Buffer is important in mobile phase optimization, since retention time depending on the molar strength of buffer.

pH of Buffer
It is maintaining pH of mobile phase in the range of 2-8 since column not withstand pH outside the range. Reason is the siloxane leakage area cleaved pH 2.00, while pH 8.00 above the silica is dissolved.[1]

Method Validation
Method validation can be defined [as per ICH Guidelines] as "Creating documented evidences, which provides a high degree of assurance that specific method or activity.

Type of method validation

A. **Full validation**
B. **Partial validation**
C. **Cross validation**

Validation Parameter
Selectivity
At least 10 sources of plasma containing heparin and anticoagulant, including of 6 normal plasma. The response of the interfering peak at the retention time of analyte should be less than 20% of mean response of the processed LLOQ sample.

Carry Over Effect
Extract and analyze blank sample, LLOQ and ULOQ in single free source of plasma. First inject LLOQ sample and after inject ULOQ Sample. The response of the interfering peak at the retention time of analyte in the blank processed sample should be less than 20% of the mean response of the processed LLOQ samples.[17]

PRECISION AND ACCURACY:

Between and within batch precision and accuracy will be calculated by analyzing three PA batches. The accuracy of at least 67% of total QC samples must be within ± 15% of the respective nominal value except at LLQC level where it should be within ±20% of the respective nominal value.[18]

Precision of QC samples at each level should be less than 15% except at LLQC level where it should be within less than 20%.

Whole accuracy of QC samples at every level should be within ±15% except at LLQC level where it should be within ±20%.[19]

RECOVERY:

$$\text{mean \% recovery of analyte} = \frac{\text{mean peak area response of analyte in extracted samples}}{\text{mean peak area response of analyte in aqueous samples}} \times 100$$

Recovery for method need not be 100% but should be precise.

The C.V. [%] of the mean analyte and internal standard recoveries must be less than 20% across all the three QC samples.[18]

Matrix Effect

It is important to consider the variability of matrix sample due to physiological nature of sample[6]. %C.V. of average peak area ratios of post spiked LQCs and HQCs should not exceed 15%.[20]

Stock solution stability

Calculated % change should be within ± 10%.

Long Term Stability

At least 67% of the total number of Quality control samples and 50% of the quality control samples at each level should be within 85-115 % of their nominal value.[17]

CONCLUSION:

This review describes various aspects of the hyphenated technique; LC MS/MS, used for the bioanalysis. Bioanalytical method development and validation are required for the information of bioavailability and bioequivalence. These studies provide pharmacokinetic, toxicokinetic and metabolic data of drugs. Bioanalytical method development consists of sample preparation, chromatographic separation and detection by using LC MS/MS. Validation of a bioanalytical method comprises of various validation parameters which includes accuracy, precision, sensitivity, selectivity, standard curve, limits of quantification, range, recovery stability, etc. LC MS/MS is most widely used analytical technique for bioanalytical method development and validation. Bioanalysis by LC MS/MS provides low detection limits, good ability to generate structural information, minimal sample requirement and wider coverage of range of analytes different in their polarities.

Conflict of interest: The authors confirm that this review article contents no conflict of interest.

ACKNOWLEDGEMENT:

The principle author acknowledges the financial assistance provided by R.C. Patel Institute of Pharmaceutical Education and Research [RCPIPER] Shirpur Dist. Dhule

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